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SHORT COMMUNICATION

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The herpes simplex virus type 1 tegument protein VP22 is known to be highly phosphorylated during infection. Here we show that two electrophoretic forms of VP22 can be identified in infected cell extracts and that this heterogeneity is accounted for by phosphorylation. Furthermore, the nonphosphorylated form of VP22 appears to be specifically incorporated into virions. We also show that the phosphorylated form of VP22 is the only form detected during transient transfection and as such that VP22 can act as a substrate for a cellular kinase. Phospho-amino acid and phospho-peptide analyses of *in vivo* labeled VP22 were utilized to demonstrate that the phosphorylation profiles of VP22 synthesized during transfection and infection are the same. In both cases VP22 was modified solely on serine residues located in the N-terminal 120 residues of the protein. Moreover, *in vitro* phosphorylation was utilized to show that the constitutive cellular kinase, casein kinase II, which has four serine consensus recognition sites at the N-terminus of VP22, phosphorylates VP22 in the same manner as observed *in vivo.* This kinase also phosphorylates VP22 at the N-terminus in intact capsid–tegument structures. Casein kinase II is therefore likely to be the major kinase of VP22 during infection.

The herpes simplex virus type 1 (HSV-1) structural protein VP22 is a major component of the virus tegument (*1*), the region situated between the capsid and the envelope of the virion. However, the role of VP22 in the infectious cycle is unclear. Several studies have suggested that VP22 can interact with HSV-1 DNA (*2–4*) and that it may associate with the nuclear matrix at some time during infection (*4*), but the relevance of these results is yet to be determined. In our attempts to define the role of VP22, we have recently shown that it interacts specifically with the acidic activation domain of VP16 (*5*), a tegument protein which functions as the transactivator of IE gene expression (*6–8*). These results indicate that VP22 may be involved in several aspects of virus replication including regulation of VP16 activity and virion assembly.

VP22 is a 38K basic protein encoded by the UL49 gene (*9*), which is located among the cluster of genes UL46–UL49, all of which encode tegument components (*10–12*). VP22 appears to be posttranslationally modified in several ways. In particular, VP22 is heavily phosphorylated in infected cell extracts (*2, 9*), but the kinase responsible for phosphorylation of VP22 is unknown. Recent *in vitro* studies have implicated the putative viral kinase, the product of UL13, in this role (*13*). However, the VP22 open reading frame contains 16% serines and threonines, many of which are contained within consensus recognition sites for cellular kinases (see Fig. 2), and thus VP22 may act as a substrate for a viral kinase, a cellular kinase, or both. In this report we describe the mapping of the region of VP22 which is phosphorylated *in vivo,* during both infection and transient transfection. In both situations the phosphorylation profile was identical, occurring only at the N-terminus of the protein, and specifically on serine residues. *In vitro* kinase assays were utilized to implicate the cellular kinase, casein kinase II (CKII), in the modification of VP22. In addition, in the context of intact capsid-tegument structures VP22 was one of only two virion proteins which acted as an efficient substrate for CKII *in vitro.* Moreover, we demonstrate that VP22 present within the virion is a nonphosphorylated form of that found in the cell, suggesting that assembling particles specifically incorporate this form of the protein.

VP22 from different sources was initially characterized by Western blotting. Total cell extracts were made from COS-1 cells either infected with HSV-1 strain 17 (m.o.i. of 10) and harvested 20 hr postinfection or transfected with the VP22 expression vector pGE109 (*9*) and harvested 40 hr posttransfection. In addition, a total extract of purified extracellular virions (strain 17), purified from infected BHK-21 cells on a 5–15% Ficoll gradient as described previously (*14*), was analyzed. The samples were electrophoresed on a 10% polyacrylamide gel, which was transferred to nitrocellulose, and reacted with the polyclonal anti-VP22 antibody AGV30, raised against a GST-VP22 fusion protein. Interestingly, VP22 synthesized during transient transfection migrated with a slower mobility

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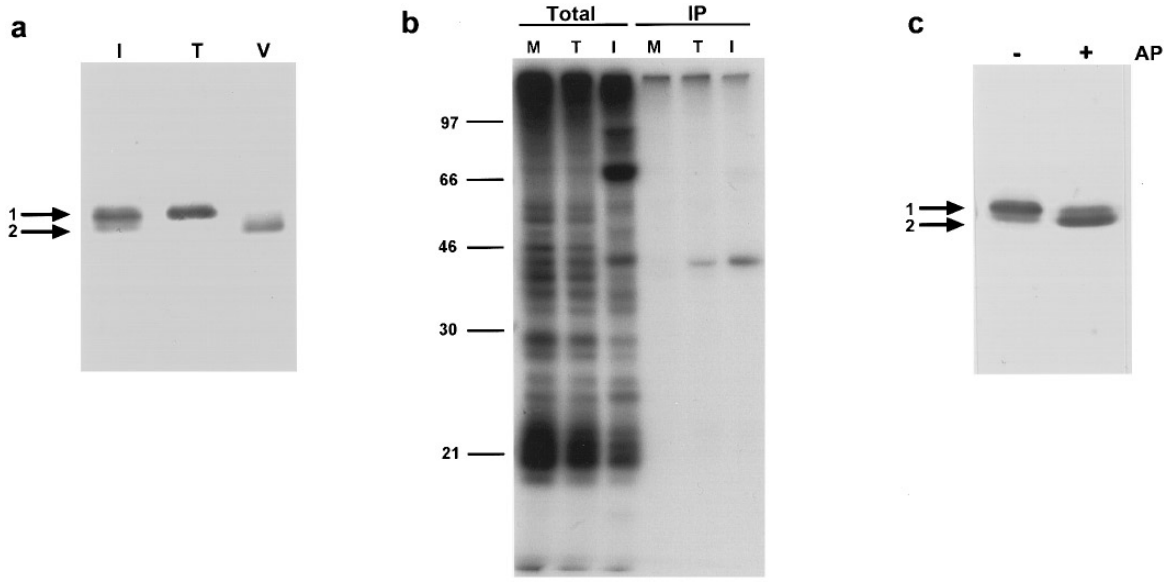

FIG. 1. (a) VP22 exists in two forms. Equivalent amounts of total HSV-1 infected COS-1 cell extract, and pGE109 transfected COS-1 cell extract were electrophoresed on a 10% polyacrylamide gel and analyzed by Western blotting, together with a sample of purified extracellular HSV-1 virions. The blot was reacted with the polyclonal anti-VP22 antibody, AGV 30. The two species of VP22 are indicated by arrows. (b) VP22 is phosphorylated in both transfected and infected cell extracts. *In vivo* ^{32}P-labeled extracts of mock, pGE109 transfected, and HSV-1 infected cells were immunoprecipitated with antibody AGV 30 and analyzed by SDS–PAGE on a 12% polyacrylamide gel. Total samples (Total) and immunoprecipitated samples (IP) are shown. M, mock; T, pGE109 transfected; I, HSV-1 infected, COS-1 cells. (c) The upper form of VP22 is phosphorylated. HSV-1 infected COS-1 cell extract was incubated for 30 min at 37° in either the absence (−) or the presence (+) of alkaline phosphatase and analyzed by Western blotting. The two forms of VP22 are indicated by arrows.

than that present in the intact virion (Fig. 1a, compare lanes T and V). Moreover, VP22 synthesized in the infected cell appeared as a broad band on the blot (Fig. 1a, lane I), ranging in migration from that seen in the virion (22^2) to that present during transfection (22^1).

The presence of VP22 species with varying mobility in our infected cell extract suggested that differentially modified forms of the protein are synthesized during infection. VP22 has previously been shown to be phosphorylated in infected cells, a modification which could account for the shift in mobility. By contrast, the phosphorylation status of VP22 synthesized during transfection, and thus in the absence of other viral gene products, has not yet been reported, but the presence of only one species in such an extract, that is 22^1, allowed us to determine the level of phosphorylation on this form of VP22. COS-1 cells, which had been either mock transfected, transfected with plasmid pGE109 24 hr previously, or infected with HSV-1 strain 17 (m.o.i. of 10) 4 hr previously, were incubated in the presence of [^{32}P]orthophosphate (50 μCi/ml) for 16 hr, harvested in PBS, and soluble proteins extracted by freeze–thawing in a buffer containing 10 m*M* HEPES (pH 7.9), 400 m*M* NaCl, 0.1 m*M* EDTA, and 0.5 m*M* DTT. An equal volume of a buffer containing 10 m*M* HEPES (pH 7.9), 0.1 m*M* EDTA, and 0.5 m*M* DTT was added to the extracts to give a final NaCl concentration of 200 m*M,* and immunoprecipitations of VP22 were carried out. Antibody AGV30 was added to the extracts, which were incubated for 2 hr at 4°. Protein A–Sepharose beads were then added and incubated for 1 hr at 4°, and the immunoprecipitated samples were washed five times in 50 m*M* Tris–HCl (pH 7.5), 150 m*M* NaCl, and 0.1% NP-40. Both total and immunoprecipitated samples were analyzed by SDS–PAGE. VP22 immunoprecipitated from infected cell extract was phosphorylated (Fig. 1b, IP samples, compare lanes M and I). Moreover, analysis of the total phosphorylated profile of infected cells revealed that VP22 represents one of the major infected cell phosphoproteins (Fig. 1b, total samples, lane I). In addition, immunoprecipitation of the transfected cell extract clearly demonstrated that VP22 is phosphorylated during transfection (Fig. 1b, IP samples, lane T). These results suggest that 22^1 represents a phosphorylated form of VP22 and therefore that VP22 can act as a substrate for a cellular kinase.

The sample of VP22 which was immunoprecipitated from infected cells appeared to contain only one ^{32}P-labeled band in its profile (Fig. 1b, IP samples, lane I), suggesting that 22^2 observed in Western blotting (Fig. 1a, lane I) may represent the unmodified form of VP22. To determine if 22^2 is a nonphosphorylated form of 22^1 a sample of infected cell extract, solubilized in the buffer described above, was treated with 5 units of calf intestinal alkaline phosphatase (AP) in 50 m*M* Tris (pH 8.5), 0.1 m*M* EDTA for 30 min at 37°, electrophoresed on a 10% gel, and analyzed by Western blotting. Treatment with AP caused an obvious change in mobility of the predominant species of VP22 in the infected cell extract from 22^1 in

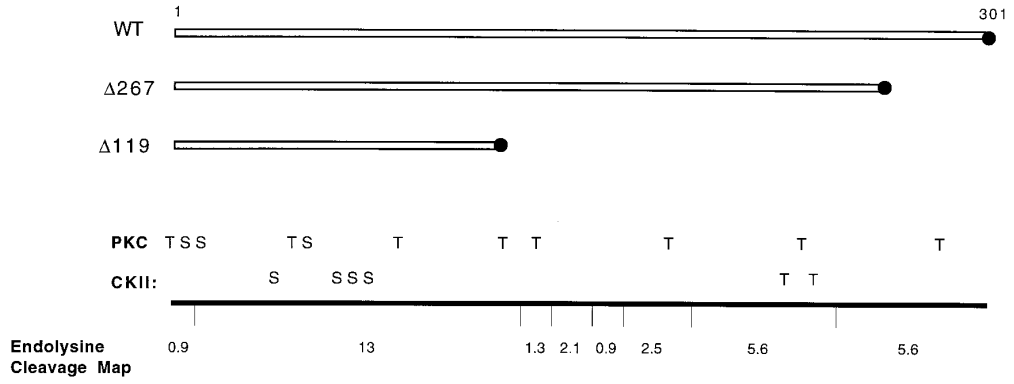

FIG. 2. Line drawing of the VP22 open reading frame (ORF). The endolysine cleavage map is shown beneath the ORF, along with the predicted M_r of the individual peptides. The relative positions of the consensus PKC and CKII phosphorylation sites are shown above the line. S, serine; T, threonine. Note that there are no consensus PKA sites. At the top of the figure are shown the open reading frames for the C-terminal deletion mutants of VP22. ●, epitope tag.

untreated extract (Fig. 1c, lane −) to 22^2 in AP treated extract (Fig. 1c, lane +). Taken together these results demonstrate that VP22 exists in two major forms within the cell—the faster migrating form (22^2), which is the species present in the virus particle and which is also detectable in the infected cell, and the slower migrating form (22^1), which represents the phosphorylated version of 22^2 and which is present in both transfected and infected cells.

While VP22 is phosphorylated in both the absence and the presence of other viral products, it is possible that different phosphorylation sites and/or different kinases are utilized in either situation. The open reading frame for VP22 is extremely Ser/Thr rich, and there are multiple consensus recognition sites for the cellular kinases CKII and protein kinase C (PKC), but none for protein kinase A (Fig. 2). To initially characterize VP22 phosphorylation, we determined the relative phosphorylation on Ser and Thr residues by phospho-amino acid analysis. *In vivo* phosphate labeling of both transfected and infected COS-1 cells was carried out as described before, and the labeled extracts were immunoprecipitated, electrophoresed, and blotted onto PVDF membrane. The ^{32}P-labeled VP22 bands were visualized by autoradiography (Fig. 3a), excised from the blot, and subjected to acid hydrolysis in 5.7 *M* HCl for 1 hr at 110° (*15*). The resulting hydrolyzed amino acids were analyzed by 2-D chromatography using unlabeled phospho-serine, phospho-threonine, and phospho-tyrosine as markers. The results of this analysis (Fig. 3b) demonstrate that VP22 from both transfected and infected cells is phosphorylated solely on serine residues.

To determine the region of VP22 which is phosphorylated in the cell, the same *in vivo* labeled VP22 was analyzed by protease cleavage. The ^{32}P-labeled, immunoprecipitated VP22 was blotted onto nitrocellulose, the VP22 band was excised and cleaved overnight in the presence of urea at 37° with 0.5 μg of endo-lysine C (Boehringer-Mannheim), as described previously (*16*). The resulting peptides were analysed by high-resolution urea/SDS–PAGE (*17*). Cleavage of both infected-cell and transfected-cell VP22 resulted in the presence of only one ^{32}P-labeled peptide with a M_r of around 20K (Fig. 3c). Analysis of the endo-lysine cleavage map of VP22 (Fig. 2) reveals that there is only one large peptide in the open reading frame which covers the N-terminus of the protein and has an apparent M_r of 13K. While the peptide we observed migrated more slowly than this, it is likely that the high level of VP22 phosphorylation would cause it to migrate aberrantly. It is noteworthy that while full-length VP22 migrates at 38K, its predicted molecular weight is only 32.2K (see also below).

The endolysine mapping of VP22 predicts that the majority of phosphorylation occurs within the N-terminal 140 residues. Thus, to confirm phosphorylation at the N-terminus, we analyzed three variants of VP22, kindly provided by John McLauchlan. Plasmids expressing full-length VP22 (UL49ep) (*18*), VP22 deleted from residues 267 to 301 (Δ267), and VP22 deleted from residues 119 to 301 (Δ119) (Fig. 2), which all contained a C-terminal epitope tag, were transfected into COS-1 cells and labeled with either [^{35}S]methionine or [^{32}P]orthophosphate. Soluble extracts were immunoprecipitated with an antibody specific for the epitope tag at the 3′ end (anti-CMV late nuclear antigen, Capricorn) and analyzed by SDS–PAGE. The ^{35}S-labeled profiles (Fig. 3d, ^{35}S samples) demonstrate that the overall level of expression of Δ119 was somewhat reduced in comparison to full-length and Δ267 variants. However, the relative levels of the ^{32}P-labeled proteins were the same as the ^{35}S-labeled proteins (Fig. 3d, ^{32}P samples), as judged by quantitation with a PhosphorImager, implying that all three proteins are equally phosphorylated. Therefore, and in agreement with the phospho-peptide mapping, the Δ119 variant of VP22 is as efficiently phosphorylated in the cell as full-length VP22, implying that the N-terminal 120 residues

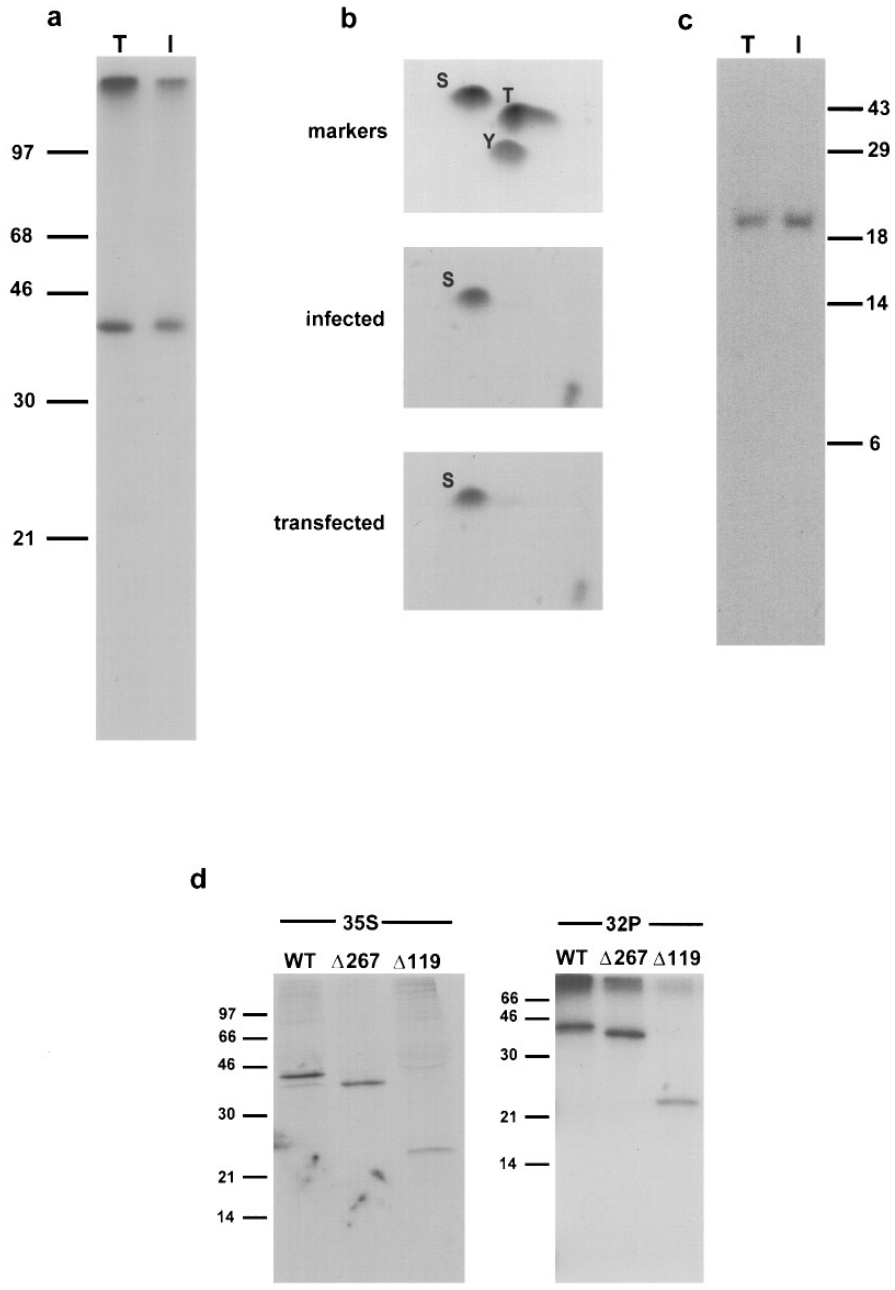

FIG. 3. VP22 phosphorylation *in vivo* is located on serine residues present in the N-terminal 120 residues of the protein. (a) Immunoprecipitation of VP22 from *in vivo* ^{32}P-labeled transfected and infected cell extracts was carried out as for Fig. 2b and analyzed by SDS–PAGE on a 10% polyacrylamide gel followed by transfer to PVDF membrane. The radiolabeled bands were visualized by autoradiography. T, transfected; I, infected. (b) The bands from (a) were excised, subjected to acid hydrolysis, and analyzed by 2-dimensional chromatography. The relative migration of cold markers is shown at the top. S, serine; T, threonine; Y, tyrosine. (c) *In vivo* ^{32}P-labeled samples were cleaved with the protease endolysine-C, digested to completion, and analyzed by high-resolution SDS–PAGE. T, transfected; I, infected. (d) COS-1 cells transfected with plasmids UL49ep (WT), △267, or △119, were labeled *in vivo* with either [^{35}S]methionine or [^{32}P]orthophosphate. The cell extracts were immunoprecipitated with the monoclonal antibody specific for the epitope tag and analyzed by SDS–PAGE on a 12% polyacrylamide gel.

of VP22 contain all the phosphorylation sites utilized *in vivo.* In addition, the △119 variant of VP22 migrates with a much slower mobility (22K) than predicted by its M_r (14K) confirming the result obtained for the migration of the 13K endo-lysine cleaved peptide.

Within the 13K peptide of VP22, which we have shown to be phosphorylated, there are four consensus PKC sites, only one of which is a serine residue, and four consensus CKII sites, all of which are serines (Fig. 2). To determine if the N-terminus of VP22 can act as a substrate for either or both of these kinases, *in vitro* phosphorylation assays of immunoprecipitated VP22 were carried out. Extracts were made from cells transfected with either full-length (UL49ep) or △119 VP22

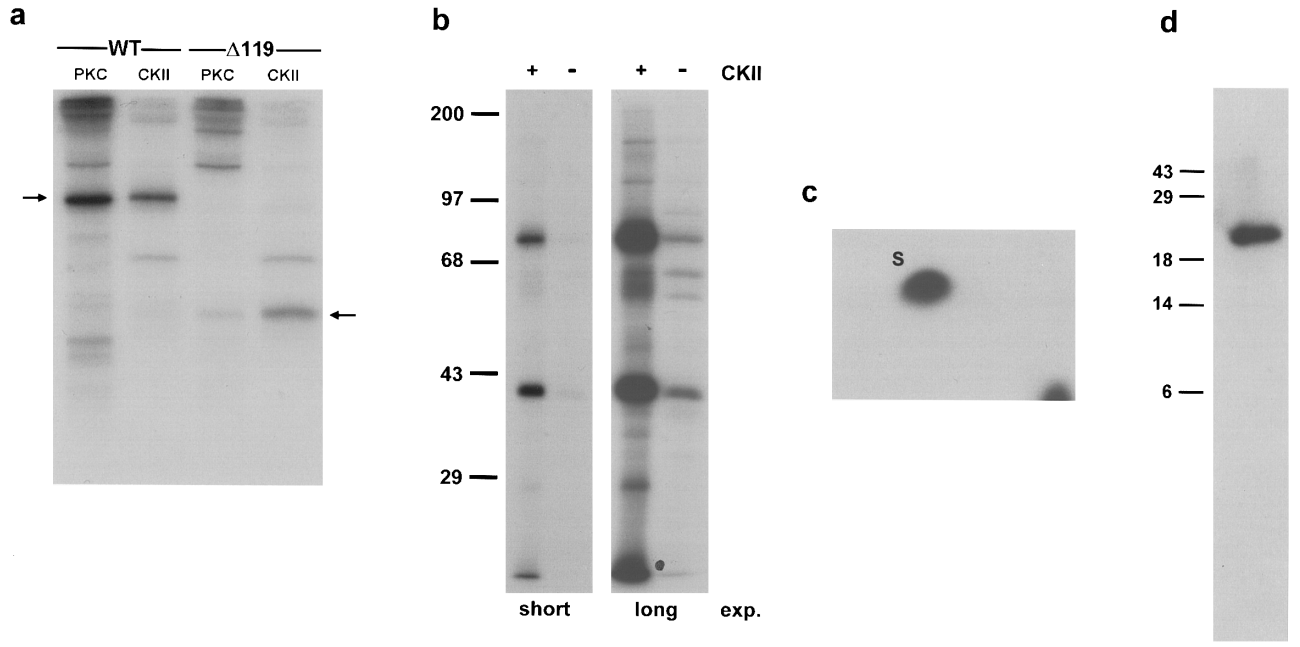

FIG. 4. Casein kinase II phosphorylates VP22 at the N-terminus. (a) COS-1 cell extracts of either UL49ep (WT) or △119 transfections were immunoprecipitated with the anti-epitope tag antibody and phosphorylated *in vitro* with either PKC or CKII. The samples were then analyzed by SDS–PAGE on a 12% polyacrylamide gel. (b) HSV-1 virions, which had been stripped of their envelopes by detergent treatment, were phosphorylated *in vitro* in the absence (−) or in the presence (+) of CKII. The resulting phosphoproteins were separated by SDS–PAGE on a 10% polyacrylamide gel. Short (2 min) and long (15 min) exposures are shown. (c) Phospho-amino acid analysis of *in vitro* CKII phosphorylated virion VP22 was carried out as described for Fig. 3b. S, serine. (d) Endolysine-C cleavage of *in vitro* CKII phosphorylated virion VP22 was carried out as described for Fig. 2c.

plasmids, and immunoprecipitation was carried out with the anti-epitope antibody. The washed samples, still bound to the Protein A–Sepharose beads, were split in two and resuspended in either CKII buffer (20 m*M* Tris (pH 7.5), 50 m*M* KCl, and 10 m*M* $MgCl_2$) or PKC buffer (20 m*M* Tris (pH 7.5), 10 m*M* $MgCl_2$, 0.5 m*M* $CaCl_2$, 100 μg/ml phosphotidylserine, and 20 μg/ml diacylglycerol). Then 10 μCi of γATP was added to each reaction along with either 166 units of CKII (New England Biolabs) or 0.08 units of PKC (Boehringer-Mannheim). The reactions were left at 30° for 30 min and the samples run on a 10% polyacrylamide gel. The results show that full-length VP22 can act as a substrate for both CKII and PKC in this *in vitro* assay (Fig. 4a, WT samples). However, while CKII phosphorylated the △119 variant of VP22 to a similar level as WT (taking into account the lower level of △119 expression), PKC phosphorylation of the N-terminus was greatly reduced in comparison to full-length (Fig. 4a, △119 samples). These results suggest that *in vitro* CKII, and not PKC, mimics the phosphorylation observed for VP22 *in vivo* (see also below), and therefore it is the likely candidate for the cellular kinase responsible for VP22 phosphorylation.

To determine if VP22 present within the virion can act as a substrate for CKII, intact virions were stripped of their envelopes by incubation for 1 hr in 10 m*M* Tris (pH 7.5), 1 m*M* $MgCl_2$, 1 m*M* DTT, and 0.05% NP-40 at 4°. The resulting capsid–tegument structures were then subjected to *in vitro* kinase treatment either in the absence or in the presence of CKII, solubilized, and analyzed by SDS–PAGE. Virions are known to have an associated kinase activity present in the tegument (UL13) (*19, 20*), such that *in vitro* phosphorylation carried out in the absence of an added kinase results in a number of phosphorylated proteins (Fig. 4b, long exposure, lane −). However, phosphorylation carried out in the presence of CKII was much more efficient than autophosphorylation (Fig. 4b, short exposure, lane +). Interestingly, only two proteins within the capsid–tegument structures acted as efficient substrates of CKII in this context—a protein of 38K, which is likely to be VP22 (see below), and a protein of 80K whose identity has not yet been determined, but which is the correct size for another tegument protein, the product of UL47. The lower phosphorylated band was confirmed as VP22 by Western blotting (data not shown). To compare CKII phosphorylated VP22 to *in vivo* phosphorylated VP22 the phospho-protein was characterized as described before by both phospho-amino acid analysis and phospho-peptide mapping. The results of these assays showed that, as for *in vivo* labeled VP22, all CKII phosphorylation was present on serine residues (Fig. 4c), and all the phosphorylated sites were present in the N-terminal peptide migrating at 20K (Fig. 4d). Thus the characteristics of VP22 phosphorylated *in vitro* by CKII are identical to those of VP22 phosphorylated *in vivo,* strongly implicating CKII as the major kinase of VP22 within the cell.

In this paper we have shown that the HSV-1 tegument

protein VP22 is phosphorylated in the cell by the cellular kinase CKII. Phosphorylation of VP22 causes a shift in its mobility such that two forms of the protein can be discerned by Western blotting, with the phosphorylated form (22^1) migrating more slowly than the nonphosphorylated form (22^2). Moreover, 22^2 accumulates in the cell only when it is synthesized during infection where it appears to be specifically incorporated into assembling virus particles and does not accumulate during transient expression. This suggests that some aspect of infected cell metabolism, possibly a specific feature, either inhibits phosphorylation or induces dephosphorylation of a population of VP22 molecules late in infection, and this nonphosphorylated form is selectively incorporated into the virion. With regard to this, we have recently shown that VP22 interacts directly with another tegument protein in infected cells, the IE-transactivator VP16, an interaction likely to be important for virus, and specifically tegument, assembly. Thus, it will be of interest to determine if VP16 interacts specifically with the lower form of VP22. The presence of an alternative phosphorylated form of VP22 implies that the role of VP22 is more than that of a structural component of HSV-1. Specific phosphorylation may control the function of VP22, for instance, by directing the protein to subcellular compartments or by specifying interactions with individual cellular or viral components. Additionally, VP22 has also been shown recently to be modified by nucleotidylylation and mono (ADP)-ribosylation (*21*), and these further modifications may act in concert with phosphorylation to control the function of VP22.

Our results presented here suggest that the major kinase of VP22 during infection is the cellular kinase CKII. This enzyme is constitutively active and is found in the cytoplasm and nucleus of all cells. A previous report has described results obtained *in vitro* which suggest that the potential virion kinase UL13 is also able to phosphorylate VP22 (*20*). However, as we did not observe any obvious differences between VP22 phosphorylated *in vivo* in transfected or infected cells, we conclude that UL13 does not play a major role in the direct phosphorylation of newly synthesized VP22, although undetectable subtle differences may be present. The UL13 gene is not essential for viral growth (*13, 22, 23*) and no direct interaction between VP22 and UL13 has yet been demonstrated. Moreover, UL13 has not been shown conclusively to act as a kinase (*13*). In addition, previous studies of *in vivo* phosphorylated cell extracts found no difference in the phosphorylation of VP22 in WT and UL13 deletion mutant infections (*22, 24*). It is therefore possible that UL13 is involved in VP22 phosphorylation indirectly, for instance by upregulating the activity of CKII in the cell.

CKII phosphorylation of envelope-stripped virions demonstrated that VP22 is one of only two proteins accessible to the kinase in this context. Availability of VP22 and the 80K protein to the kinase may suggest that these two proteins are located on the outside of the tegument. Moreover, as it is likely that the capsid–tegument structure is the one presented to the cytosol of the cell upon virus entry, these *in vitro* phosphorylation events may mimic the situation in the cell. Early phosphorylation of VP22 by CKII may provide a mechanism for dissociating it from the tegument structure, either separately or in association with VP16, enabling it to reach the correct subcellular location. Further studies will be required to determine the role of phosphorylation in VP22 function and dissociation from the tegument.

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